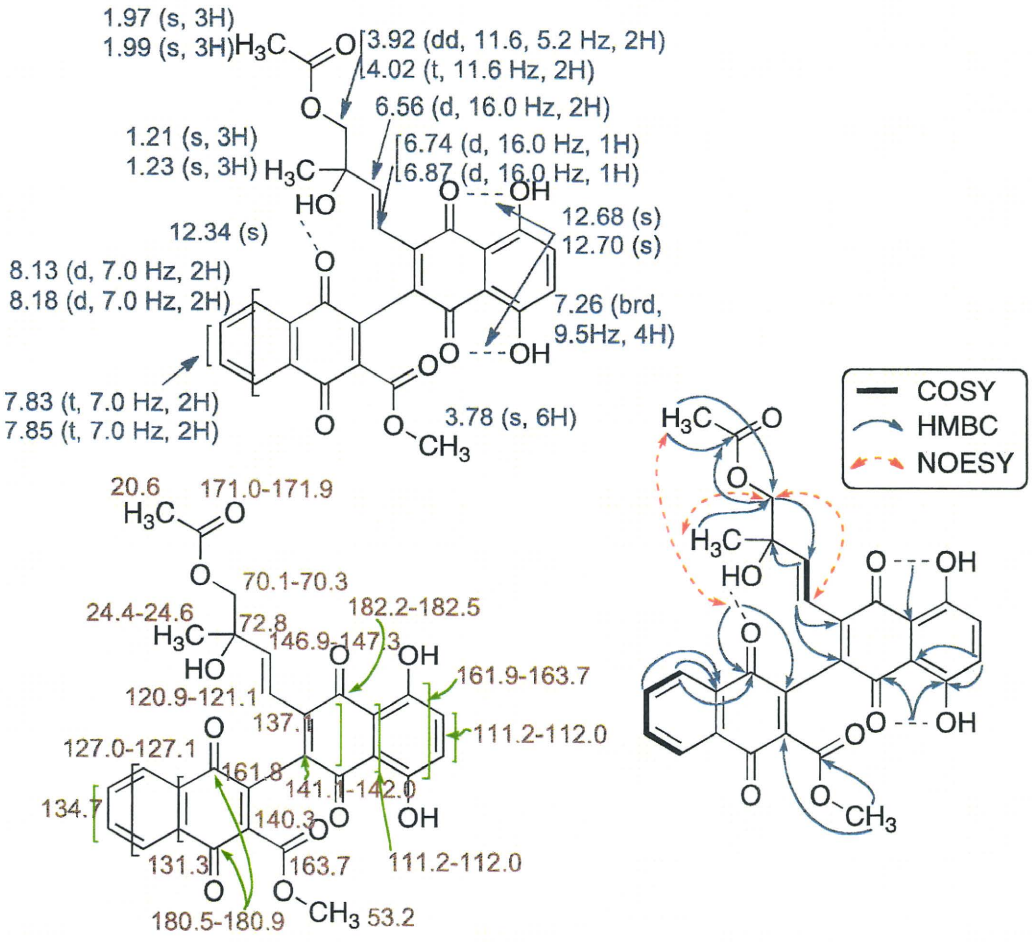

図 2　化合物 **3** の構造 (700 MHz, $CDCl_3$)

然より得られた報告は無く、新規化合物であると確認した．化合物 **1**-**2** は新規化合物であり（20 年度報告済み）、**4**-**7** は NMR 等の解析の結果既知化合物であることを確認した．また、化合物 **4**, **5** は単結晶が得られたため X 線結晶構造解析を行ったところ、既知物質と推定された (**5** の絶対配置は未決定)．これにより、**5** の結晶は三斜晶形であり非対称単位が 12 単位程度の非常に大きな結晶群を形成することが明らかになった (図 3)．

また、単離された化合物の抗リーシュマニア活性の結果を表 1 に示した．

(2) ミャンマーにて入手した3種の植物についてエキス作成し抗リーシュマニア活性試験を行った．結果を表2に示した．エキスは、現地名ドローチャ メタノールエキス、ドローチャ水エキス、トゥエアーセーメタノールエキス、トゥエアーセー水エキス、ゴーリィチャメタノールエキス、ゴーリィチャ水エキスを用いた．

D. 考察

Tectona grandis の材はミャンマーにおいて主に高級家具家具材として使用され、その葉は廃棄されている．今回行った抗リーシュマニア試験で強い活性が見出され、葉に含まれる化合物 1～4 を含む赤い色素部分に非常に強い活性があることが実験過程で明らかになった．

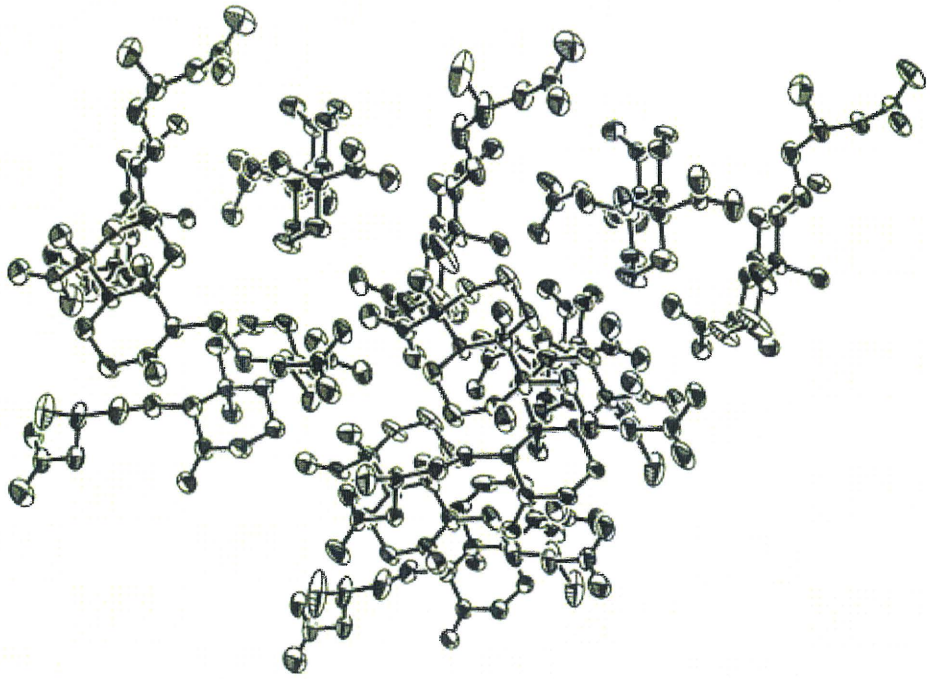

図 3　化合物 5 の X 線結晶構造解析

表1 エキスおよび単離された化合物の抗リーシュマニア活性試験の結果(μg/mL)

	Lm	Lg	Lp	Lpe
酢酸エチル分画	6.25	-	-	-
水分画	400	-	-	-
化合物 1	0.47	0.36	0.40	>25
化合物 2	9.73	3.88	8.43	>25
化合物 3	0.43	0.50	0.27	>50
化合物 4	>100	-	-	-
化合物 5	92.4	>50	21.0	>50
化合物 6	40.3	-	-	-
化合物 7	-	-	-	-

Leishmanicidal assay (in vitro): 原虫はproma-stigote体 *Leishmania major* (Lm), L. guyanensis (Lg), L. panamensis (Lp), L. peruviana (Lpe)を用いて試験を行った. 値はエキスについてはMIC (μg/mL, コントロールを)、化合物は IC50 (μg/mL)で示した. ポジティブコントロールは amphotericin B を用いた (IC50 <0.1 μg/mL).

表 2 ミャンマー産植物エキスの抗リーシュマニア活性試験の結果(MIC: μg/mL)

エキス名	Lm
ドローチャ メタノールエキス	400
ドローチャ 水エキス	>400
トゥエアーセー メタノールエキス	400
トゥエアーセー 水エキス	>400
ゴーリィチャ メタノールエキス	>400
ゴーリィチャ メタノールエキス	>400

今回の成分検索により、得られた化合物のうち、新規化合物 1 及び 3 が最も顕著な活性を示した. (IC50 043-0.47 μg/mL) また、今回得られた既知化合物である

lutein, 5-Hydroxydigitolutein, enantio-oliveric acid は抗リーシュマニア活性を示さなかった.

現在、さらに分画をすすめ化合物を単離しており、それらの構造決定ならびに活性については来年度に報告する. また、マウスマクロファージ様細胞 RAW264.7 に対する増殖抑制試験を行い、化合物の影響を検討する予定である. また、ミャンマーカチン州プタオ郡にて入手した 3 種の植物(ドローチャ、トゥエアーセー、ゴーリィチャ)は、現地で薬用として骨折や炎症を和らげる用途で使用されているものであるが、活性試験の結果、抗リーシュマニア活性は見られなかった. 現在、これらのエキスを用いて、神経様細胞 P19 の突起誘導能ならびにタンパク質凝集能について検討を行っている.

E. 学会発表

1. Yasumoto, Kanami Mori1; Nochi, Hiromi 1; Tamoto, Koichi 1; Fuchino, Hiroyuki 2; Agatsuma, Yutaka 3; Satake, Motoyoshi 3, 4; Sekita, Setsuko 1,, Study of anticancer and antiprotozoa products from tropical plants, Pacifichem 2010 (Hawaii, USA), 2010 12.

2. 黒柳正典、村田美紀、代田修、安元加奈未、関田節子、渕野裕之、中根孝久, パキスタン産 Withania coagulans の抗リーシュマニアステロイド, 第 54 回香料・テルペンおよび精油化学に関する討論会 (山梨), 2010.10.

3. 渕野裕之、川原信夫、河野真理衣、石川勉、小林正規、竹内勤、佐竹元吉、安元加奈未、黒柳正典、関田節子、Fernando Cabieses Molina、Zuno Burstein Alva: 薬用植物における抗リーシュマニア活性化合物の探索研究, 第 52 回天然有機化合物討論会 (静岡), 2010.9.

4. 紺野勝弘、数馬恒平、安藤賢司、佐竹元吉、安元加奈未、関田節子、二瓶賢一、加藤学、Marisa Rangel, Marcia Cabrera, 単独性カリバチ毒から得られた新規抗菌ペプチドの構造と生物活性, 日本生薬学会第 57 回年会 (徳島), 2010.9.

5. 菊池博之、渕野裕之、杉村康司、飯田修、川原信夫、渡邊高志、岡田稔、小山鐵夫、佐竹元吉、安元加奈未、関田節子; 抗リーシュマニア活性を有する薬用植物の探索 (その 21) -ペルー産ソロモン諸島産植物の活性評価、及び Mutisia acuminata の活性成分-, 日本生薬学会第 57 回年会 (徳島), 2010, 9.

F. 論文発表

1. In vitro leishmanicidal activity of benzophenanthridine alkaloids from Bocconia pearcei and related compounds, Chem. Pharm. Bull. 58(8), pp.1047-1050 (2010), H. Fuchino, M. Kawano, K. Mori-Yasumoto, S. Sekita, M. Satake, T. Ishikawa, F. Kiuchi, and N. Kawahara

2. Cephastigiamide A, and antiplasmodial activity of Cephalotaxus alkaloids from Cephalotaxus harringtonia form a fastigiata, Heterocycles 81(2), pp441-450 (2010), H. Morita, Y. Nagakura, T. Hosoya, W. Ekasari, A. Widyawaruyanti, K. Mori-Yasumoto, S. Sekita, Y. Hirasawa

分担研究報告書

大麻の DNA 鑑定を目的としたゲノム多様性に関する研究

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研究要旨　大麻 DNA 鑑定技術の開発研究を行った。大麻の麻薬性成分である THC(tetrahydrocannabinol、カンナビノイド化合物)を含有する「ドラッグタイプ型」大麻(アサ、*Cannabis sativa* L.) 1 種を植物材料として用いた。①「生葉」、②「乾燥葉」、③「種子」の 3 種類の検体を分析試料として、(1) *rbcL* 遺伝子および(2)ドラッグタイプ型 THCA 生合成酵素遺伝子の 2 領域の検出実験を行った。その結果、①「生葉」、②「乾燥葉」、③「種子」の全ての分析試料において *rbcL* 遺伝子、ドラッグタイプ型 THCA 生合成酵素遺伝子の 2 領域の検出が可能であることが明らかとなった。

A. 研究目的

近年わが国における違法大麻事犯の急増により、押収大麻の鑑定の必要性が増している。この場合に被疑者が有していたものが大麻であることの立証が必要である。個人で栽培、増殖したものであるのか、あるいは他者から譲り受けたものであるのかなどを立証する必要がある。また違法大麻栽培事例では、被疑者の所有していた種子や乾燥試料が大麻であるかどうかを立証する必要性がある。そこで本研究では大麻のゲノム多様性を様々な観点から解析し、DNA による系統間差、個体間差などの識別技術の開発を目的とする。特に大麻の「乾燥試料」や「種子」などを試料として DNA 鑑定を容易に行う技術を確立することが必要である。本件では①「生葉」、②「乾燥葉」、③「種子」の 3 種類の検体を分析試料として、(1) *rbcL* 遺伝子および(2)ドラッグタイプ型THCA生合成酵素遺伝子の 2 領域の検出実験を行った。

B. 研究方法

【植物材料】

大麻カンナビノイド成分である THC を含有する「ドラッグタイプ型」1 系統のアサ(*Cannabis sativa* L.)を実験材料として用いた。①「生葉」、②「乾燥葉」、③「種子」の 3 種類の検体（各 1 検体）を分析試料とした。①「生葉」は人工気象条件下（25 ℃, 24 時間連続照明）で育成した植物体(図 1)の展開葉（100 mg）を用いた（図 2)。②「乾燥葉」は人工気象条件下で育成した植物体の展開葉を乾燥機で乾燥 (60℃、一晩) したもの (20 mg) を用いた（図 2)。③「種子」は生の種子一粒（15 mg）を用いた（図 2)。

【DNA 抽出】

①「生葉葉（100mg)」、②「乾燥葉(20mg)」、③「種子(15mg)」の各 1 試料を DNA 抽出材料とした(図 2)。QIAGEN DNeasy Plant Mini Kit (QIAGEN)により添付プロトコルに従い DNA 抽出を行った。得られた DNA を 10 ng/μl に調製

図 1 人工気象条件下におけるアサの育成状況と展開葉

図 2 DNA 抽出に用いた①「生葉」、②「乾燥葉」、③「種子」試料。

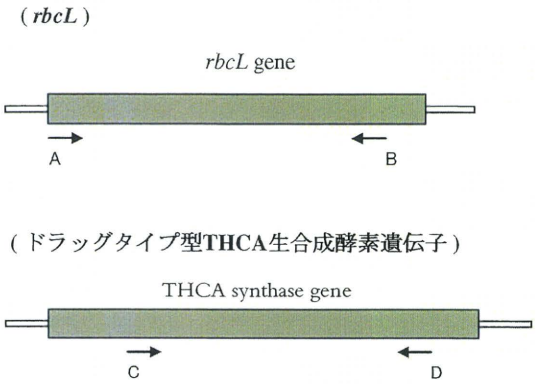

図 3 *rbcL* 遺伝子およびドラッグタイプ型 THCA 生合成酵素遺伝子の各領域に用いたプライマー部位

A:5'-TGT CAC CAA AAA CAG AGA CT-3'

B:5'-TTC CAT ACT TCA CAA GCA GC-3'

C:5'-AAT AAC TCC CAT ATC CAA GCA-3'

D: 5'-AGG ACT CGC ATG ATT AGT TT-3'

し DNA 分析試料とした。

【PCR】

(1) rbcL 遺伝子及び (2) ドラッグタイプ型 THCA 生合成酵素遺伝子の 2 領域を検出対象とした(図 3)。

(1) rbcL 遺伝子

rbcL 遺伝子は植物の光合成をつかさどる生体内酵素であるリブロースリン酸カルボキシラーゼのラージユニットをコードする遺伝子である。葉緑体ゲノム中に多くのコピー数で存在しており、PCR による遺伝子の検出が比較的容易である。ここでは rbcL 遺伝子領域を PCR 検出のポジティブコントロールとして用いた。プライマーは Hipkins et al.(1990) のプライマーA(5'-TGT CAC CAA AAA CAG AGA CT-3')、プライマーB(5'-TTC CAT ACT TCA CAA GCA GC-3')を用いた(図 3)。PCR 反応条件は 94℃ 2 分プレヒート、98℃ 10 秒、58℃ 30 秒、68℃ 1.5 分を 30 サイクルとした。PCR 反応液の一部 3 μl を 2%アガロースゲル(TAE

buffer）電気泳動後、エチジウムブロマイドで染色し 254nm で検出した。なおネガティブコントロールには蒸留水を用いた。

(2)ドラッグタイプ型 THCA 生合成酵素遺伝子

大麻麻薬性成分 THC は植物生体内で THCA（tetrahydrocannabinolic acid）として存在している。THCA は前駆物質から THCA 生合成酵素により作り出される（Sirikantaramas *et al.*, 2004）。THC を含有する「ドラッグタイプ型」大麻において、THCA 生合成酵素遺伝子を検出する手法がこれまでの研究成果により確立されている（Kojoma *et al.*, 2006）。この領域は大麻であるかどうか、あるいは「ドラッグタイプ型」大麻を検出するのに有効な領域である。プライマーは Kojoma *et al.*（2006）のプライマーC（5'-AAT AAC TCC CAT ATC CAA GCA-3'）、プライマーD（5'-AGG ACT CGC ATG ATT AGT TT-3'）を用いた（図 3）。PCR 反応は前述と同様に行った。

C. 研究結果

【*rbcL* 遺伝子、ドラッグタイプ型 THCA 生合成酵素遺伝子領域の検出】

(1) *rbcL* 遺伝子

rbcL 遺伝子領域の検出結果を図 4 に示した。①「生葉」、②「乾燥葉」、③「種子」の 3 種類の検体全てにおいて約 1.4 kb の明瞭なシングルバンドが検出された。

(2) ドラッグタイプ型 THCA 生合成酵素遺伝子

ドラッグタイプ型 THCA 生合成酵素遺伝子領域の検出結果を図 5 に示した。①「生葉」、②「乾燥葉」、③「種子」の 3 種類の検体全てにおいて約 1.2 kb の明瞭なシングルバンドが検出された。

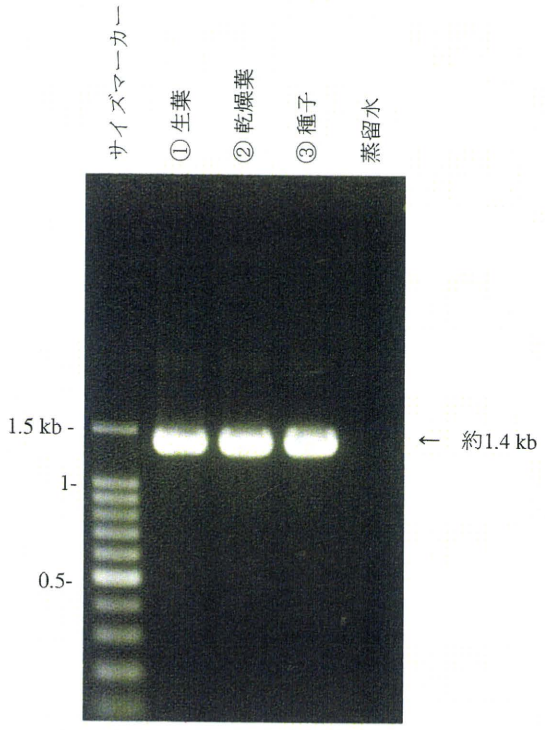

図 4 *rbcL* 遺伝子領域の PCR 断片の検出

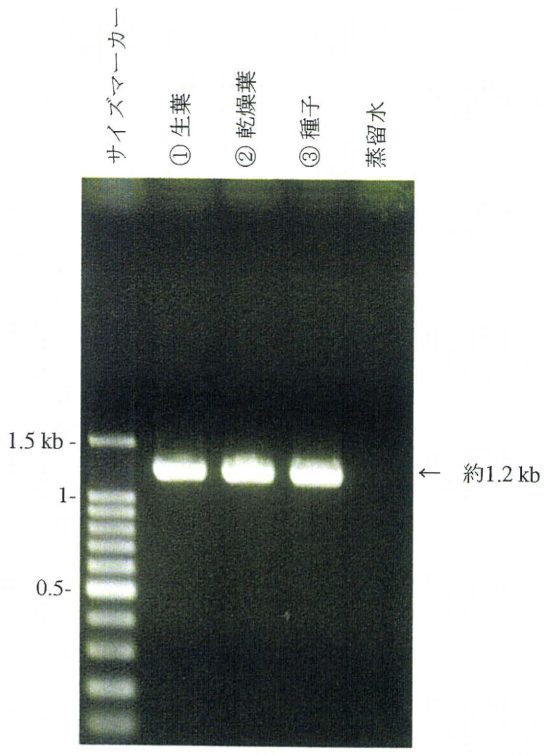

図 5　ドラッグタイプ型 THCA 生合成酵素遺伝子領域の PCR 断片の検出

D. 考察

本研究から、「ドラッグタイプ型」大麻の「生葉」、「乾燥葉」、「種子」の3種類の試料から *rbcL* 遺伝子、ドラッグタイプ型THCA生合成酵素遺伝子の2領域の検出が可能であった。このことから「乾燥大麻試料」や「大麻種子」においてもDNA鑑定が可能であることが示唆された。今後さらに多様な試料の検討を行う必要性があると考えられる。

E. 結論

本研究から大麻の乾燥葉試料および種子試料を用いても、新鮮葉試料と同様にDNA鑑定が可能であることが明らかとなった。

F. 参考文献

1. V. D. Hipkins, CH. Tsai and H. Strauss, Sequence of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from a gymnosperm, Douglas fir, Plant Molecular Biology 15 (1990) 505–507.

2. S. Sirikantaramas, S. Morimoto, Y. Shoyama, Y. Ishikawa, Y. Wada, Y. Shoyama and F. Taura, Molecular cloning and heterologous expression of Δ^1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L., Journal of Biological Chemistry 279 (2004) 39767–39774.

3. M. Kojoma, H. Seki, S. Yoshida, and T. Muranaka, DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" *Cannabis sativa* L., Forensic Science International 159 (2006) 132–140.

F. 研究発表

学会発表

1. 高上馬希重、関崎春雄、2010年5月8日、違法薬物「大麻」のDNA鑑別手法開発に関する研究、日本生薬学会北海道支部第34回支部例会（札幌）
2. 高上馬希重、関崎春雄、2010年5月8日、違法薬物「大麻（アサ）」の植物学的特徴と多様性について、日本薬学会北海道支部第134回支部例会（札幌）
3. 高上馬希重、2010年8月10日、Cannabis 大麻の植物としての多様性、生薬若手懇談会第4回勉強会（筑波）
4. 高上馬希重、2010年9月9日、違法薬物「大麻」の植物としての多様性、日本植物学会第74回大会シンポジウム（春日井）

III. 研究成果の刊行物・別刷

1. Asahina,H., Shinozaki,J., Masuda,K., Morimitsu,Y., and Satake, M. "Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences", J Nat Med (2010) 64:133-138

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ORIGINAL PAPER

Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences

Haruka Asahina · Junichi Shinozaki · Kazuo Masuda · Yasujiro Morimitsu · Motoyoshi Satake

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Abstract Species identification of five *Dendrobium* plants was conducted using phylogenetic analysis and the validity of the method was verified. Some *Dendrobium* plants (Orchidaceae) have been used as herbal medicines but the difficulty in identifying their botanical origin by traditional methods prevented their full modern utilization. Based on the emerging field of molecular systematics as a powerful classification tool, a phylogenetic analysis was conducted using sequences of two plastid genes, the maturase-coding gene (*matK*) and the large subunit of ribulose 1,5-bisphosphate carboxylase-coding gene (*rbcL*), as DNA barcodes for species identification of *Dendrobium* plants. We investigated five medicinal *Dendrobium* species, *Dendrobium fimbriatum*, *D. moniliforme*, *D. nobile*, *D. pulchellum*, and *D. tosaense*. The phylogenetic trees constructed from *matK* data successfully distinguished each species from each other. On the other hand, *rbcL*, as a single-locus barcode, offered less species discriminating power than *matK*, possibly due to its being present with little variation. When results using *matK* sequences of *D. officinale* that was deposited in the DNA database were combined, *D. officinale* and *D. tosaense* showed a close genetic relationship, which brought us closer to resolving the question of their taxonomic identity. Identification of the plant source as well as the uniformity of the chemical components is critical for the quality control of herbal medicines and it is important that the processed materials be validated. The methods presented here could be applied to the analysis of processed *Dendrobium* plants and be a promising tool for the identification of botanical origins of crude drugs.

Keywords *Dendrobium* spp. · *matK* · *rbcL* · DNA barcoding · Phylogenetic analysis

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Introduction

The genus *Dendrobium*, distributed in most Asian countries and Australia, belongs to the family Orchidaceae [1, 2], and includes 900–2000 species [2]. In China, about 50 *Dendrobium* species have long been used in traditional medicines [3]. Also in Japan, Kan-ro-in, a Kampo medicine, consists of *Dendrobium* plants, referred to as Ganlou-in in the Chinese historical prescription book, Heji Jufang. Although it has been reported that *Dendrobium* plants have various pharmacological actions, including anti-cancer, anti-oxidant, immune modulation, and vasodilation effects [4], the chemical constituents that are responsible for these activities have yet to be identified. The fact that some *Dendrobium* species have been used as a tonic for hundreds of years in China suggests that these plants have potential for modern medicinal application in Japan. Jin chai shi hu, ma bien shi hu, and tie pi shi hu are listed in the latest Pharmacopoeia of the People's Republic of China [5]. According to Flora Reipublicae Popularis

Sinicae (FRPS) vol 19 [6], the scientific names of these species are *D. nobile*, *D. fimbriatum*, and *D. officinale* (*D. candidum* auct. non Lindl. [7]), respectively.

Many kinds of processed *Dendrobium* herbs, sometimes falsely sold as a more expensive variety, such as *D. officinale*, and numerous processed *Dendrobium* herbs with vernacular names are distributed in the Asian markets, making identification of their origin species more difficult. On the other hand, it is essential that processed materials of herbal medicine are validated from the point of view of quality control. Thus, the identification of the plant source as well as the uniformity of their chemical components is critical for their use as herbal medicines.

It is known that many *Dendrobium* plants are morphologically similar, making their identification based on morphology very difficult, except during flowering, when they can be easily classified. The most popular form of the processed drug of *Dendrobium* is called "Fengdou" in Chinese, and it appears in the shape of a small coil [3]. Identifying the botanical origin of such a processed drug is difficult because during the process, the natural resource is boiled, coiled, dried, and sometimes cut into pieces. As little is known about the chemical constituents of *Dendrobium* species, chemotaxonomy has not been applied for their botanical identification. Therefore, a reliable and brief method for the identification of these plant species needs to be developed for their further pharmaceutical utilization and investigation.

Recently, molecular systematics in plants as well as other organisms has been widely used for species identification and in the determination of phylogenetic relationships. In plants, the genes for maturase (*matK*), for the large subunit of the ribulose 1,5-bisphosphate carboxylase (*rbcL*), and for the *trnH-psbA* intergenic spacer on the chloroplast genome, are often used for molecular phylogenetic analysis [8–13]. For example, in a study using 1566 specimens of orchids in Costa Rica representing 1084 species, the *matK* sequence was shown to be useful for species identification and reconstructing phylogeny [14]. In addition to these three loci, several plastid DNA regions (*atpF-atpH* spacer, *rpoB* gene, *rpoC1* gene, and *psbK-psbI* spacer) [15] are also used in plant species identification.

Furthermore, the concept "DNA barcoding" has emerged and was successfully applied for many animal groups as an efficient species identification tool (e.g. [16]). In 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group has recommended the 2-loci combination of *rbcL* plus *matK* as plant DNA barcodes, which are sequences that vary extensively between species but hardly at all within them [15]. The methodology of DNA barcording could be applied to identify species, and to provide taxonomic information in clarifying the evolutionary relevance [17, 18].

In this study, five unprocessed medicinal *Dendrobium* plants and data deposited in the DNA database of DDBJ/EMBL/GenBank were used for species identification by phylogenetic analysis. Based on the proposal of CBOL Plant Working Group, we chose the two plastid genes, *matK* and *rbcL*, to conduct our phylogenetic analyses for assessing the intra- and interspecies relationship of the *Dendrobium* species.

Materials and methods

Plant materials

Leaves or stems of medicinal *Dendrobium* plants were collected from botanical gardens, individual cultivators, and a field, which are listed in Table 1. *Dendrobium* spp. noc-a to noc-c are *Dendrobium* hybrid cultivars of *D. nobile*. *Dendrobium* spp. noc-a and b are strains that were hybridized more than once. Both have different evolutionary lineages but the details are unknown. *Dendrobium* spp. noc-c is also a hybrid cultivar but its lineage is unknown. For *D. officinale*, plant material was not available in Japan. Therefore, the data deposited in the DNA database of DDBJ/EMBL/GenBank was used.

Isolation of DNA, amplification, and sequencing

Genomic DNAs were extracted from fresh plant samples by DNeasy® Plant Mini Kit (QIAGEN). Synthetic oligonucleotides for polymerase chain reaction (PCR) primers were obtained from Nihon Bioservice (Saitama, Japan). The primer sets used for amplification of *matK* and *rbcL* gene were as follows: OMAT1F (5′-CCGTTMTSACCATATTGC-3′) and trnK-2R (5′-AACTAGTCGGATGGAGTAG-3′) for *matK* [19]; aF (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and cR (5′-GCAGCAGCTAGTTCCGGGCTCCA-3′) for *rbcL* [11] Using Ex Taq® Hot Start Version (TaKaRa Bio.), PCR was carried out with the above primer sets and DNA (approximately 5–55 ng) as a template. The PCR conditions were 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR reaction products were separated by agarose gel electrophoresis and purified by Wizard® SV Gel and PCR Clean-Up System (Promega). The purified DNA was ligated into a T-vector using pGEM®-T Easy Vector System (Promega) and *Escherichia coli* DH5α COMPETENT high (TOYOBO) was transformed with the resultant plasmid. Plasmids were isolated by illustra™ plasmid Prep Mini Spin Kit (GE Healthcare Bioscience). At least three clones were obtained for each species and used for sequencing. Sequencing was carried out by ABI 3100 Avant and 3130xl Genetic Analyzer

Table 1 Plant materials of *Dendrobium* species examined in this study

Taxon	Identifier	Source			Accession no.	
		Abbreviation	Collection site	Wild/market	*matK*	*rbcL*
D. fimbriatum Hooker	KK	fitk	Thailand, KKC	Market	AB519776	AB519784
D. moniliforme (Linn.) Swartz	SM	mot	Tochigi Pref., SMC	Wild	AB519775	AB519786
D. moniliforme (Linn.) Swartz	n/a	mom	Mikurajima, SMC	Market	AB519773	AB519788
D. monilforme (Linn.) Swartz	n/a	mok	Kagoshima Pref., SMC	Market	AB519774	AB519787
D. nobile Lindley	KK	notk	Thailand, KKC	Market	AB519772	AB519785
D. pulchellum Roxburgh ex Lindley	KK	putk	Thailand, KKC	Market	AB519778	AB519790
D. pulchellum Roxburgh ex Lindley	HB	puth	Thailand, HBG	Market	AB519777	AB519789
D. sp. noc-a	A	noca	Okayama Pref.	Market	AB519779	AB519791
D. sp. noc-b	A	nocb	Okayama Pref.	Market	AB519780	AB519792
D. sp. noc-c	A	nocc	MPG, unknown	Market	AB519781	AB519793
D. tosaense Makino	KK	totb	Kagoshima Pref., STC	Wild	AB519770	AB519782
D. tosaense Makino	KK	tosk	Shikoku, KKC	Market	AB519771	AB519783

All voucher specimens are deposited in Satake laboratory at the Ochanomizu University

KK Karasawa, Kohji; *SM* Satake, Motoyoshi; *n/a* results matched but final identification by experts only at next flowering period, *HB* The Hiroshima Botanical Garden, ID no. 5477; *A* Hybrid Cultivars of *D. nobile*; *SMC* Satake Collection; *KKC* Karasawa Collection; *HBG* Hiroshima Botanical Garden; *STC* Seki Collection; *MPG* Medicinal Plant Garden of Showa Pharmaceutical University; *unknown* unknown origins

(Applied Biosystems) using BigDye® Terminator v1.1 and v.3.1 Cycle sequencing kits (Applied Biosystems). Each procedure using the kits was carried out following the manufacturers' instructions. The nucleotide sequences of *matK* and *rbcL* reported here had been submitted to the DDBJ/EMBL/GenBank database and their accession numbers are listed in Table 1. In this study, the obtained sequence of each gene excluding the primer sequence was considered to be the full-length sequence of the genes.

DNA sequence data analysis

DNA sequences obtained from *matK* and *rbcL* were aligned with ClustalW [20]. The Neighbor-joining (NJ) method [21] was selected for the construction of phylogenetic trees. Maximum Composite Likelihood method [22] was used for computing the evolutionary distance. The output data was processed using MEGA4 [23] to draw the phylogenetic trees. A total of 1000 bootstrap replicates were calculated for the NJ tree construction [24].

Results

First, two species classified in the Chinese pharmacopoeia, *Dendrobium fimbriatum* and *D. nobile*, were chosen. The most important medicinal *Dendrobium* in China, *D. officinale*, was not available in Japan. Therefore, we used the sequence data deposited in the DNA Database of DDBJ/EMBL/GenBank for comparison. Second, *D. moniliforme* and *D. tosaense* were chosen because these were Japanese native species that have a history of medicinal use and had been exported from Japan to China in the early twentieth century [25]. Lastly, *D. pulchellum*, which is widely found in Southeast Asia as well as in India [1, 2] and considered as a medicinal *Dendrobium*, was studied.

Phylogenetic tree analysis

For the twelve samples containing the five *Dendrobium* species (Table 1), the PCR-amplified fragments of both *matK*, including parts of the *trnK* intron, and *rbcL* genes, were sequenced. The full-length *matK* gene ranged between 1870 base pairs (bp) and 1879 bp, and that of *rbcL* was 1324 bp long. The number of nucleotides in the variable site between each pair of species is shown in Table 2.

The phylogenetic tree from the *matK* sequences including parts of the *trnK* intron showed that each species formed clearly distinctive clades (Fig. 1). *D. nobile* (notk) and three hybrid cultivars of *D. nobile* formed one clade (*D. nobile* group) although a genetic distance between notk and the other species was detected. The analysis of the phylogenetic tree constructed from *matK* without the *trnK* intron revealed that species clustering was not affected by the discarded sequence (data not shown). When using the 3′-half of the *matK* sequence without the *trnK* intron, the phylogenetic tree resulted in a clustering pattern similar to that of the full-length *matK*, although the genetic variation among *D. nobile* group was absent (data not shown). To compare the sequences obtained from the present study with other sequences deposited in the DNA database, a phylogenetic tree was constructed from partial *matK*

Table 2 Number of nucleotides in the variable site between species for full-length sequences of *matK* (upper) and *rbcL* (lower)

		matK											
		fitk	notk	mot	mom	mok	putk	puth	noca	nocb	nocc	totb	tosk
rbcL	fitk	-	19	17	17	17	14	14	16	16	16	20	20
	notk	5	-	9	9	9	23	23	3	3	3	11	11
	mot	6	3	-	0	0	21	21	6	6	6	9	9
	mom	6	3	0	-	0	21	21	6	6	6	9	9
	mok	8	5	2	2	-	21	21	6	6	6	9	9
	putk	3	2	3	3	5	-	0	20	20	20	24	24
	puth	4	3	4	4	6	1	-	20	20	20	24	24
	noca	37	38	39	39	41	36	37	-	0	0	8	8
	nocb	7	2	5	5	7	4	5	40	-	0	8	8
	nocc	6	1	4	4	6	3	4	39	3	-	8	8
	totb	3	2	3	3	5	0	1	36	4	3	-	0
	tosk	3	2	3	3	5	0	1	36	4	3	0	-

Refer to Table 1 for abbreviation of samples

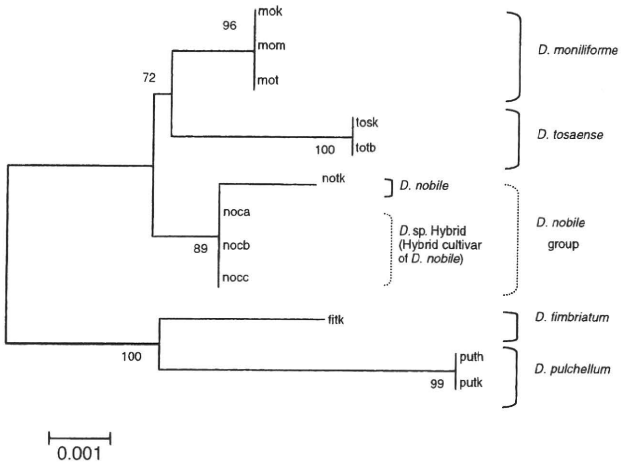

Fig. 1 Phylogenetic tree from full-length *matK* including partial *trnK* intron of 12 strains of five *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.001 nucleotide substitution per site

sequences (Nt. 537–1341, Fig. 2). In Fig. 2, most of the species formed distinct clades as shown in Fig. 1. Interestingly, sequences from *D. tosaense* and *D. officinale* excluding *D. officinale* AF445449 formed one clade.

The phylogenetic analysis using full-length *rbcL* sequences showed no species discrimination power between *D. pulchellum* and *D. tosaense* (Fig. 3). In addition, a hybrid cultivar of *D. nobile*, was not positioned within the clade of the *D. nobile* group formed from *D. nobile* and other hybrid cultivars of *D. nobile* (noc-b and noc-c). These results were also obtained in the phylogenetic tree constructed with partial *rbcL* sequences (Nt. 1–697) that were available in the database (Fig. 4). *D. officinale* (FJ216567) was located in a clade formed from *D. tosaense* but also in the clade from *D. pulchellum*.

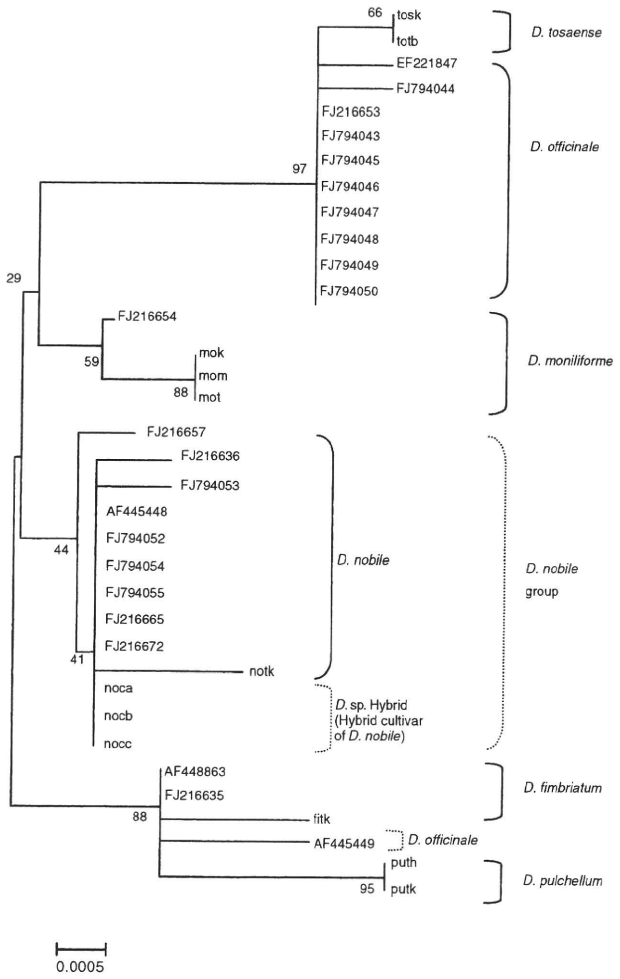

Fig. 2 Phylogenetic tree from partial *matK* (Nt. 537–1341) of 35 strains of six *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.0005 nucleotide substitution per site

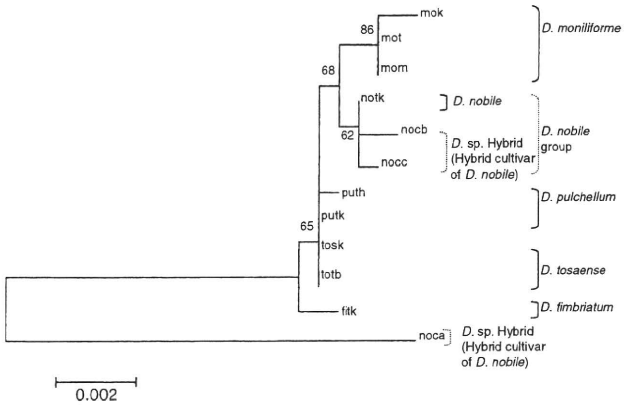

Fig. 3 Phylogenetic tree from *rbcL* of 12 strains of five *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.002 nucleotide substitution per site

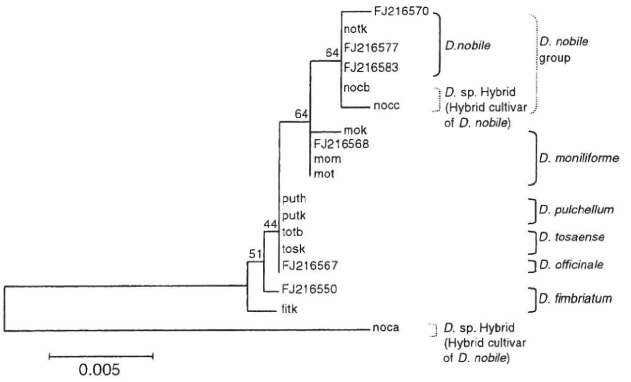

Fig. 4 Phylogenetic tree from partial *rbcL* (Nt. 1–697) of 18 strains of six *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.005 nucleotide substitution per site

Discussion

Our study showed that species identification of *Dendrobium* plants was possible using phylogenetic analyses constructed from *matK* sequences. Using the 3′-half of *matK* sequences (Nt. 944–1616), which is comparable to that of the full-length sequences, also had species discrimination power. Thus, as suggested by Lahaye et al. [14], the 3′-half of the *matK* sequence alone was useful for species identification of *Dendrobium* plants.

As shown in Fig. 1, among the species found in Honshu, Japan, *D. moniliforme* and *D. tosaense*, which seemed to have diverged later in time, were found as distinctive species in different clades. The closest species to these two species was the nobile group, although *D. nobile* formed a distinctive clade from the two species. *D. fimbriatum* and *D. pulchellum* which are morphologically categorized in the *Holocrysa* Section [1], are considered to be apart from the above three species, *D. moniliforme*, *D. tosaense*, and *D. nobile*. These three species are categorized in the *Dendrobium* Section [1]. *D. fimbriatum* and *D. pulchellum* also formed distinct clades from each other. These results demonstrate that a phylogenetic analysis using the *matK* sequence is a useful tool for the species identification of five *Dendrobium* plants.

In contrast to using the *matK* sequence, the phylogenetic tree from *rbcL* sequences had less species discrimination power. It was not possible to distinguish between *D. tosaense* and *D. pulchellum* (Fig. 3). These two species are classified as species in different sections, those of *Dendrobium* and *Holochrysa*, respectively [1]. The failure of species discrimination with the *rbcL* sequence could be due to its slow nucleotide substitution rate in comparison with other nuclear genes leading to a functional constraint that reduces the evolutionary rate of nonsynonymous substitutions [26]. From Fig. 1 and 3, we concluded that the *matK* sequence alone is probably sufficient to distinguish among these five species.

We also conducted a phylogenetic analysis of *D. officinale* and *D. tosaense* and the results pointed to the high likelihood of their being in the same clade (Fig. 2). *D. officinale* is one of the most popular and rare medicinal *Dendrobium* species in China [27], and *D. tosaense* was thought to be distributed mainly in Japan and Taiwan [28]. Based on morphological and biogeographic information, some researchers have regarded them as two different species [6, 28, 29], while Wood saw them as a common species [1]. Our phylogenetic analysis using *matK* data showed that the two species were positioned within a common clade (Fig. 2). The latest morphological and biogeographic information (refer to [2, 6, 30]) show that these two species seem to be identical and our results did not contradict this. Further sequencing of numerous data samples collected from various regions and data from traditional approaches, could finally confirm their common identity. As a whole, species identification by molecular phylogenetic analysis will help resolve the ambiguity of plant origins of crude drugs.

Regarding the *rbcL* sequence, the phylogenetic tree from the *rbcL* data showed that the hybrid cultivar, noc-a, was distantly positioned from the clade of *D. nobile* group (Fig. 3). This strain is a hybrid derived from *D. nobile* and is considered to have undergone many hybridization events, which may have caused this positioning on the phylogenetic tree.

In this investigation, the relationship between five medicinal *Dendrobium* species based on phylogenetic tree analyses constructed from *matK* and *rbcL* genes was clarified. With regard to species discrimination, *matK* rather than *rbcL* offered a higher resolution and was better suited in identifying medicinal *Dendrobium* species. Although phylogenetic analysis is a brief and rapid method, and a

powerful tool for species identification, there were still some ambiguities. Nevertheless, using a large sample of sequence data accumulated from a wide range of regions can improve the significance of the results. Overall, the use of the *matK* sequences as barcodes for the first identification process was confirmed to be very efficient. Combining data from phytochemical analysis could help standardize the species identification process of medicinal *Dendrobium* plants.

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References

1. Wood HP (2006) The *Dendrobiums*. Gantner Verlag, Ruggel
2. Baker MLB, Charles O (1996) Orchid species culture: *Dendrobium*. Timber Press, Portland
3. Bao XS, Shun QS, Chen LZ (2001) The medicinal plants of *Dendrobium* (Shi-hu) in China. Shanghai Medicinal University Press and Fudan University Press, Shanghai (in Chinese)
4. Lin P, Bi Z, Xu H, Wang Z, Xu L (2003) Advances in studies on pharmacology of plants from *Dendrobium* Sw. Chin Tradit Herb Drugs 34(11)Suppl.:19–22 (in Chinese)
5. Chinese Pharmacopoeia Committee of People's Republic of China (ed) (2005) The Pharmacopoeia of the People's Republic of China: I. Chemical Industry Press, Beijing (in Chinese)
6. Tsi Z, Chen S, Luo Y, Zhu G (eds) (1999) Flora, Reipublicae Popularis Sinicae. Angiospermae, Monocotyledoneae, Orchidaceae (3), Tomus 19. Science Press, Beijing (in Chinese)
7. Institute of Botany, Academy of Science (ed) (1976) Iconographia Cormophytorum Sinicorum Tomus V. Science Press, Beijing (in Chinese)
8. Hilu K, Liang H (1997) The *matK* gene: sequence variation and application in plant systematics. Am J Bot 84:830–839
9. Wilson CA (2004) Phylogeny of Iris based on chloroplast *matK* gene and *trnK* intron sequence data. Mol Phylogenet Evol 33:402–412
10. Kress WJ, Erickson DL (2007) A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. PLoS ONE 2:e508
11. Hasebe M, Omori T, Nakazawa M, Sano T, Kato M, Iwatsuki K (1994) *rbcL* gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns. Proc Natl Acad Sci USA 91:5730–5734
12. Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SCH (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS ONE 3:e2802
13. Steven GN, Subramanyam R (2009) Testing plant barcoding in a sister species complex of pantropical Acacia (*Mimosoideae*, *Fabaceae*). Mol Ecol Res 9(Suppl. 1):172–180
14. Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, Maurin O, Duthoit S, Barraclough TG, Savolainen V (2008) DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci USA 105:2923–2928
15. CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794–12797
16. Hebert PD, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270:313–321
17. Vogler AP, Monaghan MT (2007) Recent advances in DNA taxonomy. J Zool Syst Evol Res 45:1–10
18. Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N, Savolainen V (2005) Land plants and DNA barcodes: short-term and long-term goals. Philos Trans R Soc Lond B Biol Sci 360:1889–1895
19. Topik H, Yukawa T, Ito M (2005) Molecular phylogenetics of subtribe Aeridinae (Orchidaceae): insights from plastid *matK* and nuclear ribosomal ITS sequences. J Plant Res 118:271–284
20. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
21. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
22. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035
23. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
24. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
25. Suzuki H, Keimatsu S, Ito K (1934) Nachtrag zum "Chin-Shih-Hu". Yakugakuzasshi 54(9):820–823 (in Japanese)
26. Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84:9054–9058
27. Ding G, Zhang D, Feng Z, Fan W, Ding X, Li X (2008) *SNP*, *ARMS* and *SSH* authentication of medicinal *Dendrobium officinale* KIMURA et MIGO and application for identification of Fengdou drugs. Biol Pharm Bull 31(4):553–557
28. Kimura K (1936) Pharmacological study of Dendrobiinae plants as the Chinese drug shih-hu. J Shanghai Sci Inst Sect III 3(Suppl. 1):1–60 (in Japanese)
29. Kimura K, Migo H (1936) New species of *Dendrobium* from the Chinese drug shih-hu. J Shanghai Sci Inst Sect III 3:121–124
30. Karasawa K (2003) Species Orchidacearum I text. Japan Broadcasting Publishing, Tokyo (in Japanese)